

Identification of two novel *cis*-elements in the promoter of the prostate-specific antigen gene that are required to enhance androgen receptor-mediated transactivation

Jianye Zhang¹, Shaobo Zhang¹, Patricia E. Murtha¹, Wen Zhu², Simon S.-M. Hou¹ and Charles Y. F. Young^{1,2,*}

¹Department of Urology and ²Department of Biochemistry and Molecular Biology, Mayo Graduate School, Mayo Clinic/Foundation, Rochester, MN 55905, USA

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ABSTRACT

A monomeric androgen responsive element (ARE) is not sufficient to mediate significant androgen induction of the prostate-specific antigen (PSA) gene. Co-transfection experiments using a series of 5' deletion fragments of the proximal promoter region of the PSA gene linked to bacterial chloramphenicol acetyltransferase (CAT) as a reporter have identified two motif sequences which are indispensable for androgen receptor (AR)-mediated transactivation of the PSA promoter and have been designated as motifs A and B respectively. Of note, motif B alone has very little independent enhancer activity regardless of the presence or absence of androgen, whereas multiple copies of motif A exert androgenic inducibility for a heterologous promoter independent of the presence of ARE. Nucleotide substitutions in either motif significantly decrease the androgen inducibility and the nuclear protein binding ability. Furthermore, gel band shift experiments consistently demonstrate that nuclear proteins can bind these motifs, and they are non-receptor factors. Our data indicate that these two DNA motifs are novel *cis*-regulatory elements and exhibit different mechanisms in cooperation with ARE for AR-mediated transactivation.

INTRODUCTION

Androgens are endocrine factors that require an intracellular mediator such as the androgen receptor (AR) for their actions in target cells. Androgens play an important role in male sexual development and maturation (1) and defect or deficiency in either androgens or AR may result in a number of disorders (1-4). Moreover, androgens play a crucial role in the development and progression of prostate malignancy (5).

AR is a transcription factor that belongs to the steroid hormone/thyroid/retinoid receptor superfamily (1,6,7). Ligand-

activated AR can bind to a specific DNA sequence in order to enhance gene transcription. Therefore, the specificity of the enhanced transcription relies on at least three components. These components include ligands such as androgens, the AR and the androgen responsive element (ARE). Recent cloning and use of cDNA for AR (8-11) provide unequivocal evidence to support the above notion. Furthermore, in addition to the binding sequences for the receptors, the function of each class of steroid receptors may largely depend on the context of non-receptor binding sites in the promoter of a particular gene (1,6,12,13). Non-DNA binding proteins are also required for specific actions of the receptors via protein-protein interaction (6,14-18). However, compared to other steroid receptors, much less is known about the function of AR in this regard because fewer androgen receptor regulated genes have been characterized.

Prostate-specific antigen (PSA) is a differentiation marker for the human prostate. It has become the most sensitive marker for monitoring and detecting prostate cancer (19). Seminogelin has been suggested to be the physiologic substrate for PSA during the liquefaction process of semen coagulation (20). Recent studies have shown that PSA can proteolytically activate growth factor related proteins which might be related to the advancement of prostate cancer (19).

We and others have recently demonstrated (21,22) that the expression of PSA is mainly induced by androgens at the transcriptional level. Previous studies on mouse sex-limited protein (Slp), rat probasin, PSA and human glandular kallikrein-1 (hKLK2) genes have shown that ARE is necessary but not sufficient to confer the inducibility of gene expression by androgens (21-25). Many of the androgen regulated genes have been reported (23,25-28) to contain a complex ARE with more than one copy of ARE in the first intron or the 5' far upstream flanking region of these genes.

The Slp gene has been studied (23,24,27) in great detail with respect to the function of its AREs and the surrounding auxiliary sequences in an 120 bp DNA enhancer region. The androgen-dependent enhancer is located 2 kb upstream of the Slp gene and resides within a proviral long terminal repeat. The ARE unit in the

*To whom correspondence should be addressed. Tel: +1 507 284 9247; Fax: +1 507 284 2384; Email: youngc@mayo.edu

enhancer consists of one canonical ARE and two degenerate AREs. The degenerate AREs, unlike the canonical ARE, seem to be unable to bind AR *in vitro*. However, transfection experiments have shown that they are functional when neighboring sequences are present. Although the ARE unit decides steroid hormonal response for the SIp gene expression, the studies of Adler *et al.* (23,24) have suggested that multiple non-receptor factors binding sites are critical for determining androgen specificity. Another study has also indicated (26) that the androgen-dependent expression of a rat prostate gene requires a combinational effect of multiple AREs and other transcription factor/binding elements. Without doubt, completely defining these non-receptor factor/binding elements will help us better understand the mechanism of androgen action.

In this report, we characterize two novel non-receptor binding sequences that are required in AR-mediated transactivation of the PSA gene in human prostate cells. Gene transfer, mutagenesis and *in vitro* DNA binding assays have defined two sequences within ~15 bp in the proximal promoter of the PSA. Together with these two elements, a simple ARE exhibits significant androgen inducibility of the proximal promoter of the PSA gene.

MATERIALS AND METHODS

Plasmid constructs

To generate a series of 5' deletion fragments of the PSA promoter, PSA-624 was used as a template with a 3' oligodeoxynucleotide (ODN) and a number of 5' ODNs to produce many different lengths of PSA promoter using the polymerase chain reaction (PCR). Similar means were also used in PCR to produce PSA promoter DNA fragments containing an internal deletion or nucleotide substitution mutants. The sequences of these ODNs are listed in Table 1.

The above PCR products containing *Xba*I and *Bam*HI restriction sites at 5' and 3' ends, respectively. They were digested with *Bam*HI and *Xba*I restriction enzymes, agarose gel purified, and ligated into the vector pBLCAT3 pre-cut with *Bam*HI and *Xba*I enzymes. Double-stranded ODNs containing monomeric or multimeric region A or B sequence were inserted at *Sph*I and *Xba*I site of pBLCAT2. The above constructs were confirmed by DNA sequencing. The pBLCAT2 and pBLCAT3 contain a minimal thymidine kinase (tk) promoter and promoterless chloramphenicol acetyltransferase (CAT) gene, respectively. Unless otherwise indicated, all the ODNs used in this study were synthesized in the Molecular Biology Core Facility at the Mayo Clinic/Foundation.

Transfection experiments

Human prostate cancer cell lines PC-3 and LNCaP cells were used in transfection experiments. Briefly, cells were grown in 5% fetal bovine serum (FBS) RPMI 1640 medium at 37°C with 5% CO₂. When cells reached 50–70% confluency, they were co-transfected with designated plasmids constructed above and a human androgen receptor expression vector (kindly provided by Dr Terry Brown at Johns Hopkins University) using either DEAE-dextran-chloroquine as described previously (21) or lipofectamine according to the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD). LNCaP cells were incubated with serum-free RPMI 1640 medium 10 h before transfection. Parental vectors pBLCAT3 and pBLCAT2 were used as controls in the assays. Following transfections, cells were incubated with 1% charcoal-stripped FBS RPMI 1640 with or without 3.2 nM mibolerone (Mib; a synthetic androgen) for 24 or 48 h. Cells were then collected and extracted for use in protein assay and a two-phase fluor diffusion CAT assay as described previously (21,25). All groups of cells were prepared in duplicate for transfections, which were performed at least three times.

Table 1. Oligonucleotide sequences for PSA promoter-CAT constructs by PCR

Primers for PSA promoter construction ^{a,b}		
3' primer:	(+31/+6)	5'CTCTCCGGGTGCAGGTGGTAAGCTTG3';
5' primers:	-484:	5'GGTTGGGAGTGCAGGAAAGA3';
	-407:	5'CAGAGTGGTGCAGGGATCAGGG3';
	-371:	5'CTGAGTGCTAGTGTCTTAGGGC3';
	-342:	5'CTCTTGGAGTGCAAAGGATCTA3';
	-320:	5'GGCACGTGAGGCTTTGTATGAA3';
	-222:	5'GTCTCCATGAGCTACAAGGGC3';
	-407(A-1):	5'CAGAGTGGTACAGGGGTCAAGGGGTC3';
	-407(A-2):	5'CAGAGTGGTGCAGGGATCAGGGAGTC3';
	-407(A-3):	5'CAGAGTGGTGCAGGGGTCAAGGAGTC3';
	-407(A-4):	5'CAGAGTGGTGCAGGGGTCAAGGGGTC3';
	-407(BΔ):	5'CAGAGTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTAGTGCTAGTGTCTTAGGGCAGCTGGGTATCT-AGGCACGTGAGGCTTTGT3';
	-407(B1):	5'CAGAGTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTAGTGCTAGTGTCTTAGGGCAGCTGGGTCTT-GGCGTTCCAAGGATCTAGGCACG3';
	-407(B2):	5'CAGAGTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTAGTGCTAGTGTCTTAGGGCAGCTGGGTCTT-GGCGTTCCAAGGATCTAGGCACG3';
	-407(B3):	5'CAGAGTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTAGTGCTAGTGTCTTAGGGCAGCTGGGTCTT-GGAGTTCAAAGGATCTAGGCACG3'

^aBoth 3' and 5' primers contain *Bam*HI and *Xba*I restriction sequence at their respective 5' ends.

^bNumbers for the 3' antisense primer indicate the 5' and 3' ends respectively, relative to the cap site of the PSA transcript.

Table 2. Oligonucleotide sequences used for band-shift

Motif A and its mutants in the PSA promoter region (-396/-382) ^a :	
wild type:	5'GATCAGGGATCAGGGAGTC3' 3'TCCCTAGTCCCTCAGCTAG5'
A-2:	5'GATCAGGGGTCAAGGGGTC3' 3'TCCCCAGTTCCCCAGCTAG5'
A-3:	5'GATCAGGGGTCAAGGAGTC3' 3'TCCCCAGTTCCCTCAGCTAG5'
A-4:	5'GATCAGGGGTCAAGGGGTC3' 3'TCCCCAGTCCCCAGCTAG5'
Motif B and its mutants in the PSA promoter region (-340/-326):	
wild type:	5'GATCCTTGGAGTGCAAAGG3' 3'GAACCTCACGTTTCCCTAG5'
B-1:	5'GATCCTTGGCGTTCCAAAGG3' 3'GAACCGCAAGGTTCTAG5'
B-2:	5'GATCTCTTGGCGTTCAAAG3' 3'AGAACCGAAGTTTCTAG5'
B-3:	5'GATCTCTTGGAGTTCAAAG3' 3'AGAACCTCAAGTTTCTAG5'
PSA ARE:	5'GATCCTTGCGAAGCAAGTGCTAGCTG3' 3'GAACGCTTGTGCTTACGATCGACCTAG5'
NF- κ B ^b :	5'AGTTGAGGGGACTTCCAGGCG3' 3'TCAACTCCCCTGAAAGGGTCCG5'
AP1 ^b :	5'CGCTTGATGAGTCAGCCGAA3' 3'GCGAACTACTCAGTCGGCGTT5'
SP1 ^b :	5'ATTCGATCGGGGCGGGCGAGC3' 3'TAAGCTAGCCCGCCCGCTCG5'
hMT-IIA-MREa:	5'GATCCTGCACTCGTCGTCCA3' 3'GACGTGAGCAGCAGGGTCTAG5'

^aNumbers denote the relative position to the cap site of PSA transcript.^bNF- κ B, AP-1 and SP-1 DNA sequences were purchased from Promega (Madison, MI).

Nuclear extracts

PC-3 and LNCaP human prostate cancer cells were grown in the same conditions as described above, except that prior to nuclear extraction LNCaP cells were treated with 3 nM Mib for 12 h. Nuclear extracts were prepared as described (29). Briefly, cells were collected with Ca^{2+} , Mg^{2+} free phosphate buffered saline (PBS) containing 1 mM EDTA and centrifuged at 1000 r.p.m., 4°C for 10 min with Beckman JA 68 rotor. The cell pellet was then washed with ice-cold PBS and resuspended in 10 ml buffer containing 15 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol and 2 mM DTT and centrifuged for 10 min at 5000 r.p.m. with Beckman JA20 rotor. The pellet was resuspended in the same buffer (3 ml), homogenized by 20 strokes of a B pestle in a dounce homogenizer and centrifuged again at the same speed to remove supernatant. The crude nuclear pellet was resuspended in 0.5 ml of buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 0.6 M

KCl, 1.5 mM MgCl_2 , 0.2 mM EDTA and 2 mM dithiothreitol (DTT), 0.5 mM 2-mercaptoethanol and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and homogenized again by 40 strokes. The homogenate was incubated on icy water for 30 min with resuspension every 5 min and then spun at 15 000 r.p.m., at 4°C in Beckman JA 20 rotor for 30 min. The supernatant was collected and dialyzed against 100 vol of a buffer containing 20 mM HEPES, pH 7.5, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl_2 and 2 mM DTT, 0.5 mM 2-mercaptoethanol and 0.5 mM PMSF and 3 nM of Mib for 4 h with one change of buffer. The dialyzed nuclear extract was centrifuged at the same speed for 20 min to remove insoluble matter and stored frozen at -100°C in small aliquots. The protein concentration of nuclear extracts was measured using Bio-Rad protein assay kit (Bradford assay).

Gel band shift experiments

Double-stranded ODNs (ds-ODNs) corresponding to sequences within the A or B region in the PSA promoter and containing *Xba*I 5' protruding ends, were labeled with [α - ^{32}P]dCTP (3000 Ci/mmol; Amersham corp., Arlington Heights, IL) by Klenow enzyme to a specific activity of $8 \times 10^7 - 8 \times 10^8$ c.p.m./ μg . The sequences for these ds-ODNs are shown in Table 2.

The ds-ODNs of NF- κ B, AP1 and SP1 were purchased from Promega (Madison, WI). *In vitro* DNA binding was performed by incubating the above nuclear extract (5–8 μg) in a buffer containing 20 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl_2 , 0.1 mM EDTA, 12% glycerol, 4 mM DTT and 1 μg poly(dI:dC) with or without unlabeled ds-ODNs or a random ODN (AN_{17}) in a 100–200-fold molar excess for 10–30 min at room temperature or on ice prior to receiving 20–30 fmol of a labeled ds-ODN probe for an additional 10 min incubation.

For super-shift assay, 1 μg of specific mouse anti-AR antibody (PharMingen, San Diego, CA) was incubated with the LNCaP nuclear extract 30 min prior to *in vitro* DNA binding. Non-immune mouse IgG was included as a control. Finally, the above reaction mixtures were electrophoresed in a pre-run 5% polyacrylamide (29:1 of acrylamide:bisacrylamide) with TG buffer (12.5 mM Tris and 85 mM glycine) or 0.5 \times TBE (1 \times TBE = 0.089 M Tris-borate, 0.089 M boric acid and 2 mM EDTA, pH 8.0) at 250 V for 1–1.5 h. Gels were dried and autoradiographed.

Statistics

Student's *t*-test, one-way Anova test and Duncan's multiple range test were used for analyzing transfection data. A value of $P < 0.05$ was considered statistically significant. Mean values of CAT activities with no Mib of each construct shown in Figures 1, 2 and 4 are not significantly different as analyzed by one-way Anova with Duncan's multiple range test. Therefore, the mean values of the CAT activities without Mib in each of the above figures were treated as an equivalent background. CAT activities of each construct with Mib treatment were subtracted by CAT activities of pBLCAT3 with no Mib and used for comparison.

RESULTS

PSA glycoprotein is one of the main secretory proteins produced by the prostate. Our previous study indicated that androgens via the cognate receptor are the main factors that up-regulate the

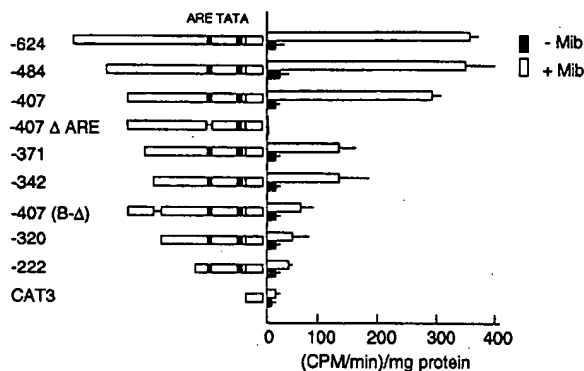


Figure 1. 5' deletion analysis of the PSA promoter using transfection assays. PC-3 cells in duplicate plates were co-transfected with designated PSA 5' deletion promoter-pBLCAT3 construct (10 μ g/plate) and a human AR expression vector (0.4 μ g/plate) using DEAE-dextran-chloroquine followed by treatment with (+) or without (-) 3.2 nM Mib for 40 h. Cell extracts were prepared from transfected cells and used for protein and CAT activity assays. The left panel of the diagram is a schematic representation of the PSA promoter fragment of each construct; numbers denote base pair position relative to the cap site of the gene. The right panel of the diagram shows the results of the CAT assay as expressed in (c.p.m./min)/mg protein. Error bars indicate the standard error of the mean of three separate experiments.

expression of the PSA gene (30,31). Although deletion of the ARE completely abolishes androgen inducibility of the proximal promoter region of the PSA gene, it seems that an ARE alone is not enough to bring about significant androgenic induction. To understand how sequences other than ARE can influence androgen inducibility, a series of 5' deletion fragments of the PSA promoter was generated (Fig. 1) for transient co-transfection with an AR expression vector in an AR lacking human prostate cancer cell line, PC-3. As seen in Figure 1, the CAT activities of constructs -624, -484 and -407 show no significant difference. The CAT activity of the -407 construct is significantly different from that of the -371 and -342 constructs ($P < 0.05$) whereas the latter two are not significantly different. The CAT activities between -342 and -320 constructs are significantly different ($P < 0.05$). Thus, there are at least two regions in addition to the ARE in the PSA promoter examined that seem to have a positive effect on AR-mediated transactivation. These two regions are at approximately -407 to -371 and -342 to -320 and designated as regions A and B respectively. Furthermore, to assure that region B contributes to androgen induction, a PSA -407 promoter construct containing an internal deletion of B (-340/-326) was made for co-transfection. As expected, lack of this region does diminish the androgen induction [407 versus 407 (B Δ), $P < 0.05$]. The third region (from -484 to -407) seemed to have some effect on the PSA promoter. However, the difference of CAT gene induction between the constructs -484 and -407 is not statistically significant. For this reason we chose to concentrate on regions A and B in the following studies.

First, to examine region A we made nucleotide substitution mutations in several positions of this region in the PSA -407 construct (Fig. 2, lower panel). Note that there are two direct repeats (i.e., CAGGGA) with one nucleotide as a spacer in this region. Three of four nucleotide substitutions are in the direct repeats. Indeed, these mutant constructs no longer exhibit androgen induction of CAT gene in the presence of activated AR

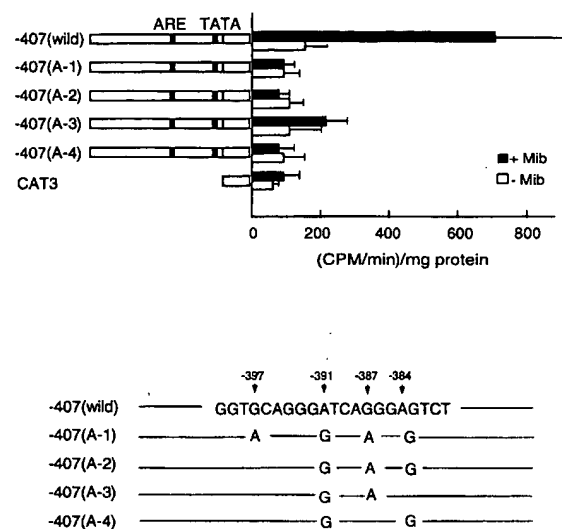


Figure 2. Characterization of the effect of mutations in region A of PSA promoter on androgen inducibility. PC-3 cells in duplicate plates were cotransfected with designated PSA promoter-pBLCAT3 constructs (4 μ g/plate) and a human AR expression vector (0.2 μ g/plate) using Lipofectamine (12 μ g/plate). Cells were then treated with or without 3.2 nM Mib for 24 h. Cell extracts were prepared and used for protein and CAT activity assays. The results are expressed in (c.p.m./min)/mg protein. Error bars indicate the standard error of the mean of three separate experiments. The lower panel represents the actual nucleotide mutations in the corresponding constructs shown in the upper panel.

(-407 versus -407A1, A2 or A4, $P < 0.05$) (Fig. 2, upper panel), suggesting that some of these nucleotides are important for the function of this region.

To demonstrate further that the DNA sequence in region A can be bound specifically by nuclear proteins, an *in vitro* gel band shift experiment was performed using nuclear extracts from PC-3 and LNCaP cells. Unlike PC-3, LNCaP cells are androgen responsive cells. We started with a 15 bp long ds-ODN encompassing -396 to -382 of the PSA promoter and similar ds-ODNs which contain the nucleotide substitutions corresponding to the constructs -407(A-2), -407(A-3) and -407(A-4) as shown in Figure 2. As seen in Figure 3, nuclear proteins can actually bind to ds-ODN -396/-382. The specificity of the protein-nucleic acid interaction is verified by competitive gel band shift assays (Fig. 3) in that the formation of 32 P-labeled ds-ODN -396/-382-protein complex is effectively blocked by excessive cold ds-ODN -396/-382, whereas mutants (Fig. 2) of ds-ODN -396/-382 have lost the ability to compete with wild type ds-ODN for nuclear protein binding (Fig. 3). The result indicates that the nucleotides within -396/-382 are sufficient for nuclear protein binding, implying that the nucleotide -397 may not be needed in both protein binding and transactivation. Both PC-3 and LNCaP nuclear extracts produce the same band shift patterns as shown in Figure 3. Using the Genetics Computer Group program (GCG; Madison, WI) program for DNA pattern search an NF- κ B-like sequence was found within the -396/-382 area, so it seemed likely that nuclear protein(s) bound to ds-ODN -396/-382 might be an NF- κ B-like factor. However, a ds-ODN containing NF- κ B sequence was not able to inhibit the formation

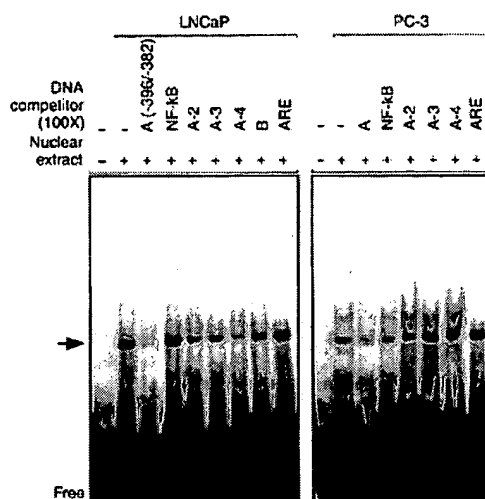


Figure 3. Band-shift analysis of prostatic nuclear protein which binds to region A of PSA promoter. 32 P-labeled double-stranded PSA -396/-382 was incubated with LNCaP or PC-3 cell nuclear extracts which had been preincubated with or without a 100-fold molar excess of designated ds-ODN under the conditions described in Materials and Methods.

of radioactive complexes. Thus, the DNA binding protein shown in Figure 3 is probably not an NF- κ B factor. In addition, PSA ARE cannot block the band formation suggesting that the DNA binding protein is not an AR. Also, an excess of B cannot compete out the protein binding of A.

Next, we wanted to further assess the sequence of region B with respect to its role in AR-mediated transactivation of PSA gene expression. Transient cotransfections were performed. In agreement with the result shown in Figure 2, a mutant with either an internal deletion or nucleotide substitutions in a 15 bp sequence in region B, except with the mutant PSA -407 B-3 (Fig. 4), exhibits a drastic reduction of androgen induction of CAT gene expression [-407 versus -407(B-1), (B-2) or (B-del.), $P < 0.05$]. It seems that the change at nucleotide -331 (G \rightarrow T) does not affect the induction [-407 versus -407(B-3), $P = 0.406$]. This implies that the change at nucleotide -334 (A \rightarrow G) is sufficient to affect the function of this area. Therefore, the transfection experiment demonstrates that the DNA sequence in region B does have a *cis*-element like function.

The result of the gel band shift experiment (Fig. 5) is essentially consistent with the findings shown in Figure 4. The formation of the PSA -340/-326-protein complexes with PC-3 cell nuclear extract can be blocked by unlabelled, homologous ds-ODN and the mutant B-3 ds-ODN but not by mutants B-1 and B-2 ds-ODNs. In addition, using other ds-ODNs including PSA-ARE and A as competitors in band shift assays the band cannot be blocked, implying that PSA -340/-326 bound nuclear protein is not AR or motif A binding proteins. The band-shift pattern was also reproducible using LNCaP nuclear extract. Moreover, DNA sequence pattern search with the GCG program found a 6 bp DNA sequence (GAGTGC) in PSA -340/-326 that is the same as the core sequence of metal responsive element a (MREa) in the promoter of human metallothionein IIA (hMT-IIA) gene (32).

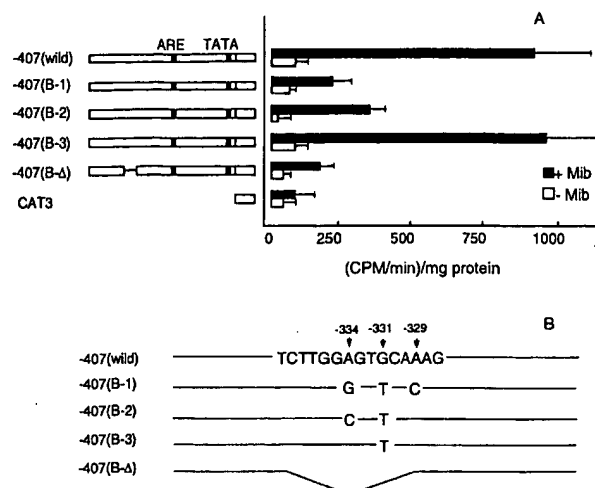


Figure 4. Effects of deletion or nucleotide substitution mutations in region B of PSA promoter on androgen inducibility. Transient co-transfection experiments were performed as described in the legend to Figure 2, except the use of PSA promoter-pBLCAT constructs as indicated in this figure. The data were collected from four separate assays. The results are expressed in (c.p.m./min)/mg protein. Error bars indicate the standard error of the mean of three separate experiments. The lower panel represents the actual nucleotide mutations in the corresponding constructs shown in the upper panel.

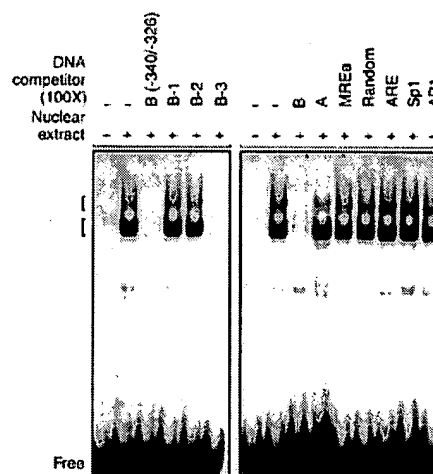


Figure 5. Interaction of prostatic nuclear protein with DNA in the region B of the PSA promoter by band-shift analysis. 32 P-labeled double-stranded PSA -340/-326 was incubated with PC-3 cell nuclear extract which had been preincubated with or without a 100-fold molar excess of designated double-stranded oligonucleotides under the conditions described in Materials and Methods.

Therefore, we were suspicious that PSA -340/-326 might contain an MRE. However, the experiment (Fig. 5) has shown this is unlikely, because hMTIIA-MREa cannot inhibit the formation of radioactive complexes.

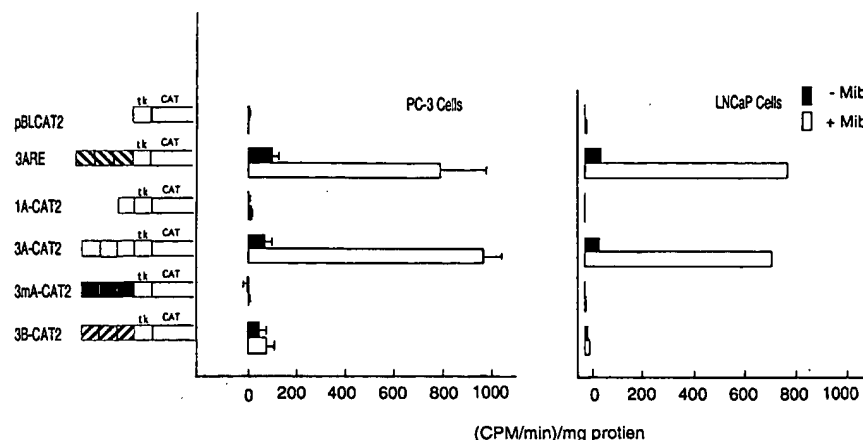


Figure 6. Androgenic induction of PSA motif A and B constructs. Transfections were performed in PC-3 or LNCaP cells either in the presence (+) or absence (–) of 3.2 nM Mib. The left panel represents the schematic promoter structure of the constructs. One or three copies of the designated motifs was inserted before tk promoter. Parental pBLCAT2 was used as a control in the transfections. The results of the CAT activity are expressed in (c.p.m./min)/mg protein. All the transfections were duplicated and repeated three times except that results from LNCaP cells were the average of two separate experiments. Error bars indicate the standard error of the mean of three separate transfections.

To further study the properties of regions A and B, a series of constructs containing monomeric or multimeric A or B were generated for the following transfection experiments in both PC-3 and LNCaP cells. As shown in Figure 6, motif B has very little transactivation activity regardless of the presence or absence of androgen, indicating that B itself may not be an enhancer motif. However, surprisingly, three copies of motif A seem to exert androgenic inducibility for a heterologous promoter without ARE, which is consistent with the result in Figure 2. The same nucleotide substitution mutation also diminishes transactivation activity of multimeric A under androgen influence. The above studies suggest that A and B motifs exert different mechanisms in assisting AR–ARE-mediated transactivation of the PSA gene. Because of the above result, we performed additional band-shift experiments to reaffirm that motif A is not a direct binding site for AR. As can be seen in Figure 7, [³²P]PSA ARE and AR from LNCaP cells can form a complex which is not only competable by unlabeled ARE but also super-shifted by a specific AR antibody. It is also evident that the super-shifted complex is able to be competed out by unlabeled ARE and that either specific antibody alone or non-immune IgG with nuclear extract does not form a complex or super-shift with the labeled ARE. In addition, the ARE–AR complex cannot be competed by a ds-ODN containing one or three copies of motif A. Conversely, the complex formed by nuclear protein and [³²P]motif A is only competable by itself but not by PSA ARE. The formation of this complex cannot be altered by anti-AR antibody. Thus, a non-AR nuclear protein forms the complex with the A motif.

DISCUSSION

PSA is one of three members of the human kallikrein gene family (19). The other two are human glandular kallikrein-1 (hKLK2) and renal/pancreatic kallikrein (hKLK1). Interestingly, both PSA and hKLK2 are expressed almost exclusively in the prostate of males and under androgen regulation. In contrast to human counterparts, rodents may have 12–24 kallikrein genes with

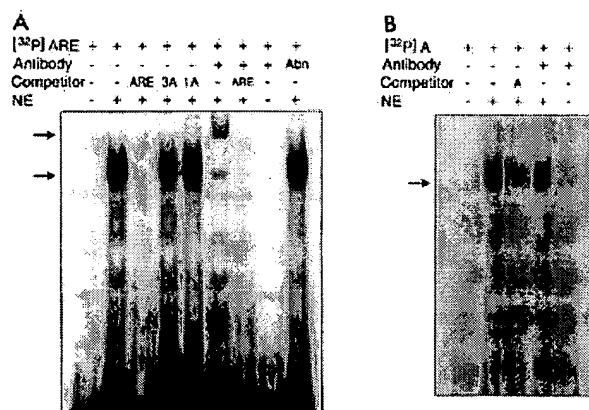


Figure 7. (A) Band shift assay of LNCaP AR and PSA ARE ³²P-labeled double-stranded ARE was incubated with 15 µg of nuclear extract proteins which were preincubated either without (lanes 2, 6, 8 and 9) or with (lanes 3, 4, 5 and 7) 100-fold of designated, cold ds-ODNs, or either preincubated with 1 µg of androgen receptor antibody (ARAb) (lanes 6, 7 and 8) or non-immune mouse IgG (lane 9). Lane 1, free probe. (B) Band shift assay of motif A binding protein using LNCaP cell nuclear extract. ³²P-labeled A ds-ODN was incubated with LNCaP cell nuclear extract (NE), which was either preincubated without (lane 2) or with 100-fold of designated, cold ds-ODNs (lane 3: A; lane 4: ARE) or 1 µg of AR Ab (lane 5) on ice for 30 min. Lane 1, free probe only; lane 6, probe A plus AR Ab but no NE.

differential expression in a variety of tissues (32). Many of these genes are also under control of steroid hormones.

Although the expression of the PSA gene is mainly controlled by androgens, intrinsic regulation may also exist because sequence analysis shows that the PSA promoter contains TATA box, CACCC box, SP1 and AP2 regulatory sequences downstream of the putative ARE. It has been demonstrated (21,22) that

a functional ARE located at -170 to -155 plays a role in androgen induction of PSA expression in prostate cells. Moreover, Riegman *et al.* (22) have inferred from their study that the region -539 to -320 in PSA promoter may cooperate with the ARE for androgen induction. The present study seems to suggest that there are at least two regions at -396 to -382 and -340 to -326 acting synergistically with the ARE for androgen induction.

The initial study, as shown in Figure 1, indicates that removal of motifs A and B by 5' deletion of the PSA promoter only causes step-wise reduction of androgen inducibility of the promoter. However, as shown in Figures 2 and 4, internal deletion mutations and most of those nucleotide substitution mutations almost completely diminish their androgen inducibility. At the present time, we cannot offer clear explanation for the discrepancy between the results from 5' deletion mutants and nucleotide substitution mutants. However, the results from the above experiments and gel bandshift assays (Figs 3 and 5) show that the sequences in regions A and B are indeed specific nuclear protein binding sites and involved in ARE/AR-mediated induction of the PSA gene. Furthermore, the aforementioned discrepancy might be an indication of a complex interaction of motif A/motif A binding protein, motif B/motif B binding protein and ARE/AR for *in vivo* androgen regulation of expression of the PSA gene.

It is generally true that multimeric interaction of homologous steroid hormone receptors or of steroid receptors and other transcription factors is required for the manifestation of steroid hormone action (6,7,12). It has been demonstrated that factors binding to SP1, NF1, OTF and CACCC-box have strong synergistic effects on progesterone or glucocorticoid receptor-mediated transactivation. Although cooperativity between different *cis*-acting elements is common, very limited information regarding cooperativity between ARE and other *cis*-acting elements is available. Among androgen-regulated genes containing functional AREs, most studies have concentrated on delineating the function of ARE. Much less attention has been paid to the detailed study of AR-cooperating factors and cognate elements.

AR/ARE is required for androgen induction of PSA and other androgen regulated genes (1). However, in order to exhibit androgen inducibility, cooperating factors/DNA elements are also needed. As shown in Figures 2 and 4, nucleotides were mutated or deleted in region A or B; subsequently, androgen induction was abolished or largely reduced even though the ARE remained intact. Gel band shift assays further demonstrated that these two regions were nuclear protein binding sites. The binding patterns produced by using PC-3 or LNCaP cell nuclear extract are the same. The result from the band shift assays suggests that these two DNA binding proteins exist commonly in prostate cells. Since PC-3 cells do not produce AR and PSA, and ARE cannot compete out the band formation by motif A or B with nuclear extracts in the band shift assays, we can conclude that the sequences in the regions A and B are two different *cis*-acting elements which are not AR binding sites. Moreover, those nucleotide substitution mutations seem to demonstrate the importance of certain nucleotides in these regions for protein binding and transactivation functions. Our study certainly warrants more detailed and comprehensive investigation into the role of nucleotide sequences in the above regions for protein binding.

It has been shown that many protein factors can interact with steroid hormone receptors and may influence receptor-mediated transactivation. Some of these factors are known DNA binding

transcription factors such as c-Fos, c-Jun and octamer factor (12,33-35). They exhibit interference or enhancement effects on receptor-mediated transactivation. Others have been shown to be associated with the receptors in a ligand-dependent manner. These receptor associated factors include the estrogen receptor-associated proteins ERAP160, RIP160 and RIP80 (36,37), the thyroid hormone receptor interacting proteins Trips and TRIP1 (38), the human homolog of the adaptor Sug1p (39), a mouse bromodomain-containing protein, TIF1 (40), the human steroid hormone receptor coactivator-1 (SRC-1) (34) and an AR-specific coactivator, ARA70 (18). In addition, the insulin degrading enzyme is also a known non-receptor, non-DNA binding factor that can directly interact with the androgen receptor protein (41). However, its role in the AR-mediated transactivation of specific genes remains to be elucidated.

Data presented in this paper implies that nuclear proteins for motifs A and B can interact with AR either directly or indirectly and enhance AR's transactivation function in a ligand-dependent manner. Moreover, it is very intriguing that motif A and its binding protein show androgenic inducibility in the presence of ligand activated AR but independent of ARE. Our observation seems to suggest that the function of motif A and its binding protein may present a novel mechanism by which AR can activate transcription rates in the absence of an ARE. This implies that if the promoter of a particular gene possesses suitable number of motif A or A-like sequences, the expression of such a gene would be regulated via ligand-activated AR at transcriptional levels.

Of note, these two motifs have little basal enhancer activities suggesting that these motifs and their binding proteins may act as accessory factors to AR. Additionally, in transfection experiments multi-copies of motif A exert androgenic induction independent of ARE, whereas multi-copies of motif B cannot. Indicating that the mechanisms by which the two motifs influence AR's function may be different from each other. Isolation of these proteins and their cDNAs in the future will greatly facilitate the understanding of how both protein-protein interaction and DNA-protein interaction influence the AR-mediated transactivation.

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